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Hemin as a generic and potent protein misfolding inhibitor

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ABSTRACT

Protein misfolding causes serious biological malfunction, resulting in diseases including Alzheimer's disease, Parkinson's disease and cataract. Molecules which inhibit protein misfolding are a promising avenue to explore as therapeutics for the treatment of these diseases. In the present study, thioflavin T fluorescence and transmission electron microscopy experiments demonstrated that hemin prevents amyloid fibril formation of kappa-casein, amyloid beta peptide and α-synuclein by blocking β-sheet structure assembly which is essential in fibril aggregation. Further, inhibition of fibril formation by hemin significantly reduces the cytotoxicity caused by fibrillar amyloid beta peptide *in vitro*. Interestingly, hemin degrades partially formed amyloid fibrils and prevents further aggregation to mature fibrils. Light scattering assay results revealed that hemin also prevents protein amorphous aggregation of alcohol dehydrogenase, catalase and γs-crystallin. In summary, hemin is a potent agent which generically stabilises proteins against aggregation, and has potential as a key molecule for the development of therapeutics for protein misfolding diseases.

1. Introduction

Most proteins typically fold into unique three-dimensional structures in order to become biologically active [1,2]. However under stress conditions (elevated temperature, extreme pH, oxidisation etc.), native proteins can misfold via partially structured intermediates to either disordered amorphous aggregates or ordered amyloid fibrils [3]. Amorphous aggregation occurs by a relatively fast and random process [4–6], whereas amyloid fibril formation occurs in a more ordered manner at a slower rate [7]. Protein misfolding which results in aggregate formation can lead to serious biological consequences. An example of amorphous aggregation is cataract, caused by misfolded crystallin proteins in the eye lens. Age-dependent post-translational modification, such as deamination, oxidation, glycation, and truncation [8–11] of lens crystallin proteins lead to their amorphous aggregation and subsequent precipitation [12] which therefore impair vision. Amyloid fibril formation is associated with more than 20 diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease [13–18]. In AD, the most prevalent age-related neurodegenerative disorder, two proteins aggregate to form amyloid fibrils, namely the amyloid-beta peptide (Aβ) and hyperphosphorylated tau protein [14,19]. In PD, α-synuclein (αS) is found to be the main protein in amyloid fibrils present in Lewy's body deposits [20–22].

To date, considerable effort has been dedicated to discovering efficacious molecules to combat protein misfolding in order to prevent these diseases or delay their onset. However, there is still no effective, widely used therapeutic to treat protein misfolding diseases. Hemin, the oxidised form of heme, is a crucial component of many physiological processes including electron transport and redox chemistry, and is essential to the function of a number of proteins, such as haemoglobin, cytochrome, catalase and peroxidase [23,24]. A previous report has shown that hemin prevents Aβ aggregation and reduces cytotoxicity of aggregated Aβ on neuroblastoma cells [25]. However the selectivity and mechanism of hemin as a protein misfolding inhibitor are still unclear. The aims of this research are (1) to evaluate the general efficacy and mechanism of hemin as a protein misfolding inhibitor; (2) to explore the properties of hemin in breaking down preformed, or partially formed fibrils of Aβ42; (3) to investigate the ability of hemin to rescue SH-SY5Y cells from toxicity associated with

Abbreviations: Aβ42, amyloid-beta peptide 1-42; AD, Alzheimer's disease; ADH, alcohol dehydrogenase; αS, alpha-synuclein; CD, circular dichroism; DTT, 1,4-dithiothreitol; RCM-κ-CN, reduced and carboxymethylated kappa-casein; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PD, Parkinson's disease; TEM, transmission electron microscopy; ThT, thioflavin T.

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amyloid fibrils; and (4) to examine the ability of hemin to prevent amorphous aggregation *in vitro*. Therefore this work will provide significant insight into the possibility of developing hemin as an effective therapeutic for preventing or treating protein misfolding diseases.

2. Materials and methods

2.1. Materials

κ-casein (κ-CN) (Sigma, USA) was reduced and carboxymethylated as previously described [26,27]. The Aβ peptide 1-42 (Aβ42) was purchased from Anaspec (USA), dissolved in 60 μL of 1.0% NH_4-OH and brought to a final concentration of 250 μM using MilliQ water. This stock solution was separated into aliquots and stored at −80 °C until use. α-Synuclein mutant A53T (A53TαS) was expressed and purified as previously described [28]. Hemin, alcohol dehydrogenase (ADH) and catalase were from Sigma. All protein solutions were prepared in phosphate buffer (10 mM, pH 7.4) and passed through a 0.22 μm syringe filter (Pall Corporation, USA) to remove any aggregates prior to experiment. Thioflavin T (ThT), (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and 1,4-dithiothreitol (DTT) were obtained from Sigma–Aldrich (Australia). Uranyl acetate was obtained from Agar Scientific (UK). Strong carbon coated 400-mesh nickel grids used for all transmission electron microscopy (TEM) imaging were purchased from ProSciTech (Australia). RPMI1640 powder, foetal bovine serum, horse serum and L-glutamine were purchased from Thermo Electron Corporation (Australia). All other reagents were of analytical grade.

2.2. Thioflavin T assay

ThT fluorescence was measured on a Fluostar Optima plate reader (BMG Labtechnologies, Australia) with a 440/490 nm excitation/emission filter set. The ThT assay was prepared in a 96-well micro-plate in duplicate and incubated in the presence of 10 μM ThT with shaking for A53TαS and without shaking for reduced and carboxymethylated κ-CN (RCM-κ-CN) and Aβ42. 10 μM A53TαS, 25 μM Aβ42 and 25 μM RCM-κ-CN were prepared in 100 mM phosphate buffer pH 7.4 in the absence and presence of 1:1 M ratio of hemin.

2.3. Transmission electron microscopy

Samples for TEM were prepared by applying 5 μL of protein solution directly from the ThT assays to 400-mesh carbon coated nickel grids, washing three times with 10 μL filtered MilliQ water, then negatively staining using 5 μL 2% (w/v) uranyl acetate. The samples were viewed using a Philips CM100 transmission electron microscope (Philips, The Netherlands).

2.4. Circular dichroism spectroscopy

All far-UV-circular dichroism (CD) spectra were acquired on a Jasco-715 spectropolarimeter at 25 °C, using a cuvette of 1 mm path length at a scan speed of 10 nm min^{-1} and a time constant of 0.125 s. Each sample (final concentration 10 μM) was prepared in phosphate buffer (10 mM, pH 7.4). The spectra were recorded in millidegree units over a wavelength range of 190–250 nm then converted and plotted as a function of ellipticity.

2.5. Methyl tetrazolium bromide assay

SH-SY5Y cells were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium containing 10% v/v horse serum, 5% v/v foetal bovine serum, 10 U mL^{-1} of penicillin and 10 μg mL^{-1} of streptomycin and maintained at 37 °C in a humidified incubator with 5% CO_2. Cells were plated at a density of 2×10^4 cells per well in 96-well plates in 100 μL full-serum fresh medium. After 24 h, the cells were treated with Aβ42 from the ThT fluorescence assay which was incubated in the absence and presence of hemin, to give a final Aβ42 concentration of 1 μM. Each treatment had six replicates. After a further 48 h of incubation, the treated cells were tested for viability by the MTT assay [29] using a BMG Polarstar microplate reader (BMG Labtechnologies, Germany). The results of the MTT assay were statistically analysed using one-way analysis of variance (ANOVA) followed by a Dunnett's comparison test (GraphPad PRISM V6). Differences were accepted as statistically significant at $p < 0.05$.

2.6. Light scattering assay

Light scattering assays were monitored at 360 nm in a Fluostar Optima plate reader (BMG Labtechnologies, Australia) at 40 °C. Samples for light scattering assays were prepared in a 96 well clear microplate in duplicate, with each well containing 200 μL protein solution either in the absence or presence of a 1:2 M ratio of hemin.

3. Results and discussion

3.1. Hemin prevents amyloid fibril formation by RCM-κ-CN, Aβ42 and A53TαS

Although amyloid fibril formation is often linked to the onset or progression of a variety of diseases, many non-disease-related proteins can also assemble into amyloid fibrils under appropriate conditions. RCM-κ-CN readily forms amyloid fibrils under physiological conditions *in vitro* [30], and has proven to be a convenient fibril-forming protein to screen for anti-amyloid compounds due to its robustness and high reproducibility [30]. In the present work, the generic anti-fibril activity of hemin was initially tested on RCM-κ-CN using a ThT assay. ThT is a benzothiazole dye that exhibits enhanced fluorescence upon binding to β-sheet rich structures, and hence is commonly used to monitor amyloid fibril formation [31,32]. As shown in Fig. 1A1, the ThT fluorescence profile of RCM-κ-CN incubated in the absence of hemin increased in intensity and reached a plateau after approximately 20 h. When RCM-κ-CN was incubated in the presence of a 1:1 M ratio of hemin, the ThT fluorescence did not increase with time.

Next, we measured the ability of hemin to prevent the PD and AD related proteins, A53TαS and Aβ42 respectively, forming fibrils. As shown in Fig. 1B1, the ThT profile of A53TαS incubated in the absence of hemin increased in fluorescence intensity and reached a plateau at 80 h. Similarly, in the absence of hemin, the ThT fluorescence intensity of incubated Aβ42 reached the plateau phase after 6 h (Fig. 1C1. Incubation with hemin prevented ThT fluorescence and hence fibril formation for both disease related proteins.

The increased ThT fluorescence intensity in Fig. 1 indicates that amyloid fibrils are formed after incubation for the three proteins studied, which is consistent with TEM images where long mature fibrils are observed (Fig. 1A2, B2, and C2). The ability of hemin to prevent fibril formation is also confirmed by TEM images where small aggregates are instead observed, as shown in Fig. 1A3, B3, and C3.

From these experiments, hemin is shown to prevent a range of peptides/proteins from aggregating to fibrils, and in doing so, converts them into small amorphous aggregate states.

The potency of hemin to prevent RCM-κ-CN fibril formation was compared with that of EGCG, which is a widely accepted